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# bFGF and PDGF: Overview and Impact on Clinical Application

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## INTRODUCTION

Wounds are one of the most widespread human afflictions. A good analogy to a wound is the common cold; everyone gets one sooner or later, and the body usually heals normally on its own, but no one really understands exactly how. For thousands of years, wound healing has been the subject of a variety of treatments that have included mud packs, honey, and fire (1, p. 1064). The presence of a handful of growth factors at the wound site led to the discovery that they are responsible for every step of the healing process, from the inflammatory response to the erection of a barrier to infection. The underlying mechanisms that control the actions of the growth factors, however, are still not perfectly understood.

I became interested in growth factor activity primarily through my research under the tutelage of Dr. Jeff MacCabe, a professor of zoology at the University of Tennessee, Knoxville. Although my research does not deal directly with wound healing, I have been able to show that basic fibroblast growth factor (bFGF) increases the survival rate of cultured 3T3 fibroblast cells (2, unpublished). As I plan to enter medical school in the fall of 1993, I am also particularly interested in growth factors because of their possible clinical applications in alleviating pain, helping to heal stubborn chronic wounds, or even speeding up the normal healing process itself. Additionally, the disruption of the growth factor mechanism is tied to a number of pathologies. Perhaps the further elucidation of the growth factor mechanism might lead to possible new treatments for these diseases. Finally, the possibility for both clinical application and financial gain has led to cutting-edge research and cut-throat competition among biotechnology companies. Some of the questions I will attempt to answer in this paper are: what are the specific actions of platelet-derived growth factor (PDGF) and bFGF; how, when, and where are they elicited; what are their mechanisms for the transduction of their signal to the recipient cell; and what are their impacts on the clinical applications of synthetic growth factors. I will also discuss societal issues such as the production of synthetic growth factors, the ethics behind the right to patent them, and where the future of growth factor research might be directed.

## METHODS

The primary sources of information that I used in researching and producing this paper were identified with the use of Medline and General Science Index versions of CD-ROM databases. Additional information was gathered using the on-line card catalog from Hodges Library at the University of Tennessee, Knoxville, and through personal conversations with Dr. Jeff MacCabe, professor of zoology, UTK, and Dr. John Koontz, professor of biochemistry, UTK.

## HISTORY OF GROWTH FACTORS

In the most general sense, growth factors can be anything that causes biological cells to grow. Specifically, the growth factors bFGF and PDGF discussed in this paper are biological proteins that are synthesized by cells and give a pleiotropic response. Their actions include the initiation of cell proliferation, or mitogenesis, an increase in cell migration, or chemotaxis, cell differentiation, and a general increase in cell and tissue survival (3, p. xi).

The first growth factor activity discovered was that of nerve growth factor (NGF) in 1952 by Rita Levi-Montalcini who described "the widespread presence of a factor endowed with nerve growth" associated with mouse sarcomas (4, p. 1156). In the next two years NGF was isolated from snake venom by Stanley Cohen (4, p. 1156). Both Cohen and Levi-Montalcini shared the Nobel Prize in 1986 for their work with NGF and epidermal growth factor (EGF) (5, p. 111). Since their initial discovery more than forty years ago, intense research has led to the description of more than 100 growth factors today (6). Some, like NGF, were discovered because their synthesis was stuck "on" and were found by analyzing sarcomas and other uncontrolled growth formations. The growth factor genes may also mutate to produce oncogenes. In fact, some growth factors are only known by their oncogenes (7). Still other growth factors are discovered by physical force. That is, if a serum is known to have a particular effect on cells, then parts of the serum are removed until the effect can be attributed to a specific component of the serum. Careful analysis of the extracellular fluid taken from healing wounds has also led to the discovery of some growth factors (8, p. 223). Once isolated, growth factors are named for where they were discovered. For instance, PDGF was first found in platelets and bFGF was first found in fibroblast cells. Their names do not make much sense anymore, however, since they are now known to be found just about anywhere in the body in some concentration (7).

## BIOLOGICAL BACKGROUND

With the ever-increasing number of growth factors that have been described, and the amount of research presently being done, similarities begin to emerge among the growth factors so that they can often be grouped into families. The primary method of grouping growth factors into families is by comparing their amino acid sequences. For instance, the bFGF family includes seven members that have similar amino acid sequences even though they are all from different genes. The type of receptor used to bind a growth factor and transduce its signal is also a characteristic which separates growth factors into families. Every member of the FGF family and the PDGF family have receptors with an intrinsic tyrosine kinase activity.

Additionally, growth factors may be characterized as either progression or competency growth factors depending on how they affect gene expression (9, p. 2). Progression growth factors are those that "stimulate genes as a result of transit through the cell cycle (9, p. 2)." That is, they simply keep the cell cycle moving, resulting in continual, uninterrupted mitogenesis.

Competency growth factors, on the other hand, are those that express genes that ultimately cause cells to be kicked out of their dormant G0 stage and into the G1 and through the S phase (9, p. 2). Another characteristic of competency growth factors is that they function even in the presence of a protein inhibitor like cycloheximide because they require no protein synthesis to transcribe "early-intermediate response" genes (6). Protein synthesis is required in the recipient cell, however, in order to activate secondary or "delayed response" genes (6). Both PDGF and bFGF are competency factors.

**Overview of bFGF : discovery and specific actions**

The FGF family consists of seven related polypeptides of which basic FGF and acidic FGF are the best characterized. bFGF was not identified until the mid 1970s (3, p. xi). Labeled first as a mitogen, bFGF has been found to produce cell proliferation in a variety of cell types from mesenchymal to neuroectodermal (10, p. 161). Figure 1 (11, p. 293) shows that exposure to bFGF causes a significant increase in arterial endothelial cell replication. Some of bFGF's activities related to its mitogenic properties include an increase in the survival of cells packed

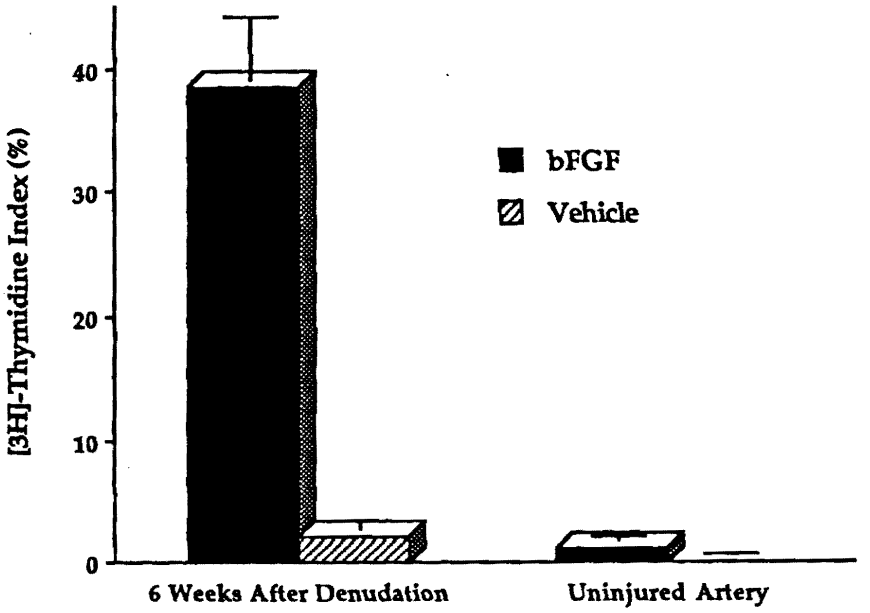


Figure 1

at clonal density, the prevention of death when cells are exposed to a stressful environment (a serum-free medium), and the general increase in the life span of cultured cells (12, p. 2). Perhaps bFGF's most distinguishing *in vivo* activity is its stimulation of angiogenesis, or the production of new blood vessels. Experiments with bFGF showed for the first time that a growth factor could be both a mitogen and a morphogen (12, p. 2). *In vitro* experiments have indicated that bFGF elicits signals in the early developing *Xenopus* embryo which stimulates the forma-

tion of mesoderm. bFGF's ability to reorganize the extracellular matrix by inhibiting collagen type I and fibronectin with various proteases and collagenases, and promoting collagen type II synthesis explains its capability to differentiate cell lines such as vascular endothelial cells and chondrocytes (12, p. 3). To summarize, bFGF's *in vivo* roles include mitogenesis, cell differentiation, embryonic development/mesoderm formation, and angiogenesis. In general, bFGF functions in the maintenance and survival of certain tissues (3, p. xi).

The bFGF gene includes two introns (16kbs each) and three exons (36kbs total) (13, p. 109). Following transcription of the gene, the mRNA is translated into a polypeptide about 154 amino acids long (14, p. 157) that encodes a complex of four isoforms (13, p. 114). The three largest isoforms are initiated unconventionally by the leucine codon (CUG) while the smaller isoform is initiated by the usual methionine "start" codon (AUG). This unusual translation is unique for bFGF and does not occur with other competency factors (15, p. 204). The recent discovery of this anomaly could mean that some activities previously attributed to the known smaller isoform could actually be functions of one or more of the larger isoforms. The differences in weight could also be a possible mechanism for post-translational regulation of expression, function, or localization of bFGF (13, p. 114). Until recently, it was thought that since two cysteine residues are conserved in all FGF family members, they must form a disulfide bond, which would also have an impact on bFGF's structure/function relationship. X-ray crystallography has confirmed, however, that no disulfide bond exists (16, p. 107).

**Secretory signal sequence, localization, and release of bFGF**

In general, growth factors are synthesized only when needed and not stored anywhere, but bFGF may be a partial exception to this rule (7). Since bFGF is the only member of its family not to have a known signal sequence for secretion, it was thought that bFGF was only released from dead or dying cells as part of the inflammatory response during wound healing (7). However, new lines of evidence suggest that there must, indeed, be a signal sequence for secretion. If one living cell is isolated and cultured, bFGF is released from the cell without the occurrence of injury (15, p. 206). Also, the differences in weight of the bFGF isoforms is shown to have a role in its localization and release. The N-terminal amino acid sequence of the heavier isoforms is called the "nuclear targeting sequence" and determines that they will be localized to the nucleus (15, p. 205). The lighter isoform, however, is localized to the cytoplasm and exported to the surface (13, p. 118). This difference in localization also suggests a difference in function. The heavier isoforms are implicated in an autocrine mitogenic action since they are localized to the nucleus, and the lighter isoform in a paracrine action since it is exported. It is known that endogenous bFGF is not enough to cause the cell to proliferate, so exogenous bFGF must be taken up (17, p. 132).

Since there is no evidence for the traditional pathway of secretion, which includes packaging by the endoplasmic reticulum and Golgi apparatus, and finally exocytosis across the membrane, a non-traditional pathway must exist. One suggestion is that bFGF binds to hep-

arin sulfate in the cytoplasm, and is transported to the membrane (18, p. 207). Another difference between bFGF and other growth factors is that it is not released in a soluble form, but is thought to become an integral part of the extracellular matrix once it is transported across the membrane. In the matrix, bFGF binds with high affinity to heparin sulfate proteoglycans (HSPGs), which serve to protect the growth factor from degradation, to keep the growth factor localized, and to keep bFGF from continually acting on itself (12, p. 4). bFGF is stored within basement membranes and in the matrix and is normally sequestered in emergencies (18, p. 208). Evidence for this kind of release is substantial. bFGF is known to be released from the extracellular matrix by heparanases (platelets, macrophages, neutrophils, or lymphocytes—cells of the immune system) which are also elicited in response to wound healing (18, p. 212). Additionally, the hydrolysis of the extracellular matrix produces active heparin/bFGF complexes. Moreover, where hydrolysis occurs *in vivo*, there is a corresponding increase in mitogenic activity (12, p. 5).

**Receptor and signal transduction for bFGF**

Two types of receptors, low and high affinity, have been described for bFGF. As previously discussed, once bFGF has crossed the membrane into the extracellular matrix, it binds to HSPGs. These heparin-like structures, which are generated by plasmin and indirectly activated by bFGF itself (19, p. 180), may also exist on the cell-surface. They exist as low affinity receptors, but do not function in transducing the bFGF signal. The HSPGs bind bFGF and induce a conformational change so that it has even higher affinity for the primary receptors (16, p. 99). Although the affinity for HSPGs in the extracellular matrix is relatively high and the abundance of HSPGs is much greater than the abundance of primary receptors, the affinity for the primary receptors is two to three times stronger (19, p. 177). The cell-associated HSPGs also act as a buffer by slowly releasing bFGF to its receptors over a period of time. Thus the bFGF is efficiently transported to its high-affinity receptor, and long-term responses can be elicited from a short burst of bFGF (19, p. 179). HSPGs also protect bFGF from degradation by proteases including trypsin and chymotrypsin (19, p. 179).

X-ray crystallography has generated a great deal of information about the structure of bFGF and its binding domains to both the receptor and the heparin substrate. The sequence of amino acids 115-124 is implicated in receptor binding because it lies on the surface of the protein and contains a Threonine-121, which is shown to enhance bFGF's affinity for the receptor when phosphorylated by protein kinase A, probably by inducing a conformational change (16, p. 99). Important residues for high biological activity of the heparin binding to bFGF are stabilized by noncovalent interactions at both the carboxy (C) and amino (N) terminal sequences (16, p. 106). There is also some evidence that heparin and the receptor might share binding site domains, but an intact 3-D structure seems to be more important to binding than actual binding domains.

Three high-affinity bFGF receptors, which do function in transducing the signal, have been isolated and described based on their possession of tyrosine kinase (T-K) domains. They are the fms-like gene (flg), the bacterially expressed kinase (bek), and the chicken embryo kinase (CEK-2) (10, p. 161). T-K receptors are characteristic of all competency factors (15, p. 204). Although bFGF binds to a receptor with T-K activity as PDGF does, its signal transduction mechanism is not identical and is not well understood.

It is thought to be similar to PDGF's mechanism in that it activates the phosphatidylinositol pathway, which is known to have an integral part in cell proliferation, but unique in that it activates the pathway at different points (7). Unlike the PDGF pathway, proliferation still occurs when the phosphatidylinositol pathway is blocked, indicating the presence of an additional mechanism (7). The pleiotropic actions of bFGF are known to be primarily regulated by the protein tyrosine kinase that is intrinsic in all of FGF receptors (20, p. 147). However, bFGF's mitogenic effects are much more efficient in combination with G-protein activating agents, like thrombin (20, p. 146). It has been demonstrated that hormones or neurotransmitters that are known to use the G-protein pathway to transduce their signals also function as mitogenic inhibitors or stimulators (21, p. 90). The application of Pertussis toxin, which is known to uncouple the G-protein pathway, succeeded in blocking hormone or neurotransmitter produced proliferation, but had no effect on growth factor mitogenesis (21, p. 91). These two lines of experiments with the phosphatidylinositol pathway and G-protein coupled pathways indicates the possibility of the existence of two independent mechanisms for bFGF signal transduction leading to cell proliferation.

**Overview of PDGF : discovery and specific actions**

In 1955, it was discovered that no animal cells could survive without a minimum amount of animal protein like blood serum (9, p. 3). Fibroblast cells that were deprived of the serum entered the G0 latent stage, but if they were restimulated with serum, they entered the G1 phase and proceeded through the cell cycle. The fact that the change induced in the fibroblast cells continued to be detectable even after the serum was removed and that the cells were committed to the change even after a brief exposure was sufficient evidence to speculate that there was some macromolecule in serum that induced the change (9, p. 3). It was not until 1977, however, that researchers discovered that the removal of platelets from the serum suspended cell proliferation (9, p. 4). This breakthrough quickly led to the isolation of the growth-inducing factor, PDGF, from platelets.

Similar to bFGF, functions of PDGF include proliferation, chemotaxis, matrix production, and an integral part in the wound healing process (22, p. 41). PDGF has also recently been found in the human placenta and in the *Xenopus* embryo which suggests a role in embryonic development and differentiation as well (22, p. 41). Sources of PDGF include platelets, endothelial cells in blood vessels, and macrophages (23, p. 1564).

PDGF is a 30 kDa disulfide-bonded dimer of two chains, A and B, which show 60% homology (22, p. 42). Combinations of these two chains can give AA, AB, and BB isoforms of PDGF, all of which have been reproduced synthetically (24, p. 328).

**Secretory signal sequence, localization and release of PDGF**

The synthesis and release of PDGF is well documented and generally understood to occur via the normal pathway. That is, PDGF is not synthesized until it is needed, not stored anywhere within the cell or the matrix, and is packaged for secretion by the endoplasmic reticulum and the Golgi apparatus directly after synthesis. Like the isoforms of bFGF, the presence of multiple isoforms of PDGF implies that they, too, might have differences in function. Indeed, there is an exception to the secretory pattern just described. AA and AB isoforms are processed via the normal pathway and secreted right after synthesis, suggesting a paracrine function (22, p. 42). Isoform BB, however, stays associated with the producer cell, which implies an autocrine function (22, p. 42).

**Receptors and signal transduction for PDGF**

Although the signal transduction through the PDGF receptor is a relatively well described system, its mechanism still has not been completely elucidated. Like the ligand, the PDGF receptor also has two similar but distinct subunits, alpha and beta. Three dimeric receptor types, each with specific ligand binding affinities, can be formed by the combinations of the two subunits. The alpha-alpha receptor type can bind any of the three isoforms of PDGF while the alpha-beta receptor can only bind AB and BB forms and the beta-beta receptor can only bind the BB form of PDGF (24, p. 336).

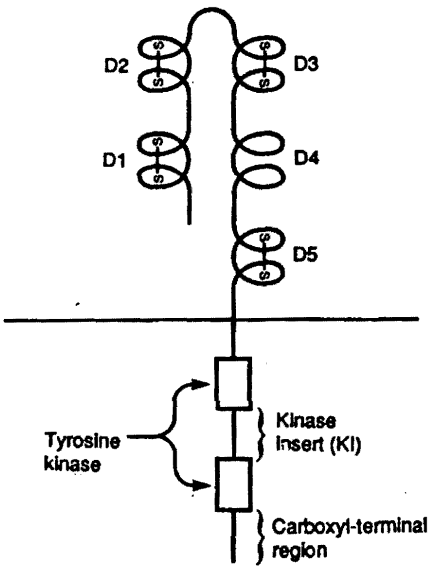


Figure 2

The PDGF receptor is a 180-190 kDa glycoprotein and can be found in the membranes of vascular smooth muscle cells, fibroblasts, and glial cells (23, p. 1564). Figure 2 (23, p. 1565) shows the organization of the PDGF receptor. The most characteristic aspect of the receptor is its tyrosine kinase domain. A unique part of the receptor is the tyrosine-kinase insert which interrupts the T-K domain. Since many growth factors do not have a tyrosine kinase insert domain, it had been suggested that perhaps it did not have a significant role in cell proliferation. However, further studies showed that the removal of the insert produced a "blunted mitogenic response (23, p. 1569)." There are also five extracellular immunoglobulin-like domains (D1-D5) (23, p. 1565). Evidence for their existence is the presence of five regular repeats in the amino acid sequence, characteristic disulfide bonds, a similarity to sequences of known immunoglobulin domains, and a capacity for recognition (23, p. 1566). The N terminus of the receptor is extracellular and the C terminus is intracellular. There is also one hydrophobic sequence of amino acids that forms the transmembrane sequence. Although its activity is not precisely known, it does serve more than just an anchor in the membrane. Replacement of the sequence with another transmembrane receptor sequence does not affect binding affinity, but fails to propagate the signal to the intercellular T-K domain (23, p. 1566). Since the AA isoform of PDGF does not stimulate chemotaxis whereas the AB and BB forms do, a difference between the alpha-alpha receptor and the other two is suggested (7, 43). Indeed, when compared, there is only a 30% homology in the extracellular domain, an 80% homology at the kinase domain, and a 30% homology at the kinase insert and carboxy terminus (22, p. 43).

As shown by Wilson in figure 3 (23, p. 1567), there are four possible models for PDGF signal transduction. The first model suggests that a conformational change is induced in the receptor by the agitation of the transmembrane portion. The second mechanism involves the perpendicular movement of the receptor to the membrane. The third mechanism is that PDGF binding may induce a receptor dimer that could result in the formation of a cytoplasmic active site for a T-K substrate. In the fourth model the receptor is already in the form of a dimer and PDGF binding induces conformational changes (23, p. 1567). The dimeric models are the most plausible since they can account for the specificity and conservation of the transmembrane portions (23, p. 1567).

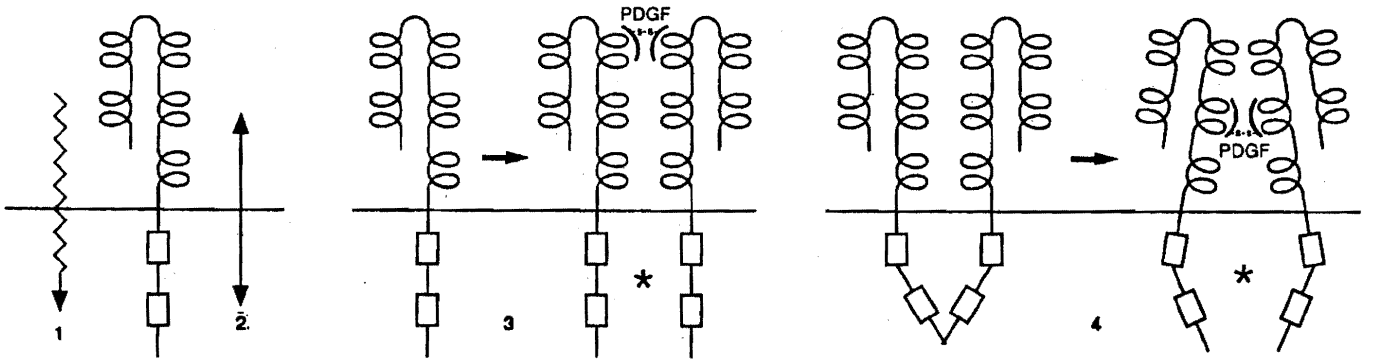


Figure 3

When PDGF binds to its receptor, a number of responses are elicited, such as activation of the T-K domain which proceeds to activate several other intracellular pathways, conformational change due to autophosphorylation, increased expression of secondary genes, change in ion fluxes, modulation of cellular pH, increase in calcium concentration, elevation of cAMP, and the downregulation of the receptor (23, p. 1568). It is possible that each of these events does not activate some independent pathway, but may all act together to provide a better environment for more efficient signal transduction (25, p. 120), replication of the DNA, and division of the cell (23, p. 1564).

It has been suggested that the T-K domain may act as a "switch kinase" that would integrate the various stimuli and relay the signal in a pleiotropic response (21, p. 87). The activation of the T-K domain is indeed critical to the transduction of the signal because it activates a number of other pathways, such as the phosphatidylinositol pathway, by phosphorylation that are known to stimulate mitogenesis. Mitogenesis due to the actions of PDGF is known to be inhibited when the phosphatidylinositol pathway is blocked (7). Autophosphorylation, or phosphorylation by a neighboring catalytic domain, of the T-K intrinsic enzyme increases the Vmax of the kinase, which enables the receptor to maintain a high level of activity even in the absence of the growth factor (26, p. 68). Although specific T-K substrates have not been identified, studies of inactivation of the T-K domain show that it is responsible for the activation of all the responses to PDGF binding except downregulation (23, p. 1568). This activity is apparently controlled by the insert domain (23, p. 1568). Downregulation is when the cell becomes less sensitive to the growth factor binding because of the degradation of previously bound receptors (6). The rate of PDGF receptor turnover is relatively high and it is not recycled back to the surface of the membrane (22, p. 43). Therefore, response to PDGF relies on the growth factor's concentration as well as the expression of the receptor. Additionally, the characteristic secondary or delayed response genes produced as a result of PDGF binding have been identified as mediators of the signal sequence. C-myc is a nuclear-binding protein and may be active as a transcriptional promoter or activator of DNA synthesis itself (9, p. 18). C-fos is a nuclear phosphoprotein involved in transcription regulation and c-jun is an important transcription factor (9, p. 18).

**What happens after the signal is transduced?**

After signal transduction takes place, most receptor-growth factor complexes are known to be taken into the cell via endocytosis and degraded by lysosomes. This is the process that contributes to downregulation. Some receptors like the insulin receptor are transported back to the membrane surface after endocytosis and reused. There is also recent controversial evidence that bFGF is taken into the cell via endocytosis as before, but is then translocated directly to the nucleus (6). The mechanism is unclear, but there is a close relationship between an increase in the amount of bFGF in the nucleus and the onset of the S phase of the cell cycle when DNA is replicated (17, p. 132).

## CLINICAL APPLICATIONS IN WOUND REPAIR

A handful of growth factors, including bFGF and PDGF, are present in the wound environment (27, p. 359). Here they are known to be essential in just about every aspect of the normal wound healing process including the restoration of blood flow, tissue strength, and a barrier to infection (28, p. 306). The normal wound healing process can be divided into three distinct stages.

The first stage in the process of wound healing is characterized by inflammation and hemostasis. A wound usually results in hemorrhaging, which is the disruption of the vascular supply. In response, platelets are mobilized to the wound site, resulting in the release of growth factors, thromboplastin, and the formation of a clot (29, p. 341). The second stage of wound healing occurs when macrophages, and fibroblasts, stimulated by growth factors, migrate to the wound site to form granulation tissue and a new layer of epithelialization (29, p. 341). The final stage of the healing process is characterized by the remodeling of the newly synthesized granulation tissue and the matrix. This process, which serves to increase the strength of the tissue, is brought about by the increase in the amount of collagen type I in the extracellular matrix (29, p. 341).

Since growth factors have been shown to be an integral part of the wound healing process, perhaps the application of synthetic growth factors could help heal chronic wounds or even speed up the process of normal wound healing. Only in the last decade, however, has there been sufficient biochemical characterization of growth factors and their activities to proceed with the application of recombinant growth factor products to the wound healing process (28, p. 306).

### Studies of bFGF's potential for clinical application

bFGF was one of the first growth factors to be evaluated as a potential synthetic product, because of its known mitogenic and angiogenic properties, both of which are extremely important in the wound healing process. The advent of Synergen's acquisition of a patent for recombinant bFGF led to a dramatic increase in the number of *in vivo* wound healing experiments.

One such experiment, as demonstrated by Broadley and Davidson (28, pp. 306-315), involved wound healing in normal juvenile rats. Three wound models were used, each providing different quantitative measurements of the healing process. The first model involves wounds that are produced by subcutaneous implants of alcohol sponges. This wound model measures the increase in the cellularity of newly synthesized granulation tissue by comparing relative content of collagen (by amount of hydroxyproline), DNA, and protein. In the second wound model, transcutaneous, longitudinal, incisional wounds are made on the backs of the rats and closed by wound clips. The third model involves a transecting wound through the median collateral ligament of the hind limb. Both the second and third models measured such

tensile properties of wound healing as breaking energy and strength. bFGF was administered on the third day after wounds were made to allow the inflammatory response to subside. Additionally, each wound was examined histologically to insure that mitogenesis and angiogenesis were indeed occurring.

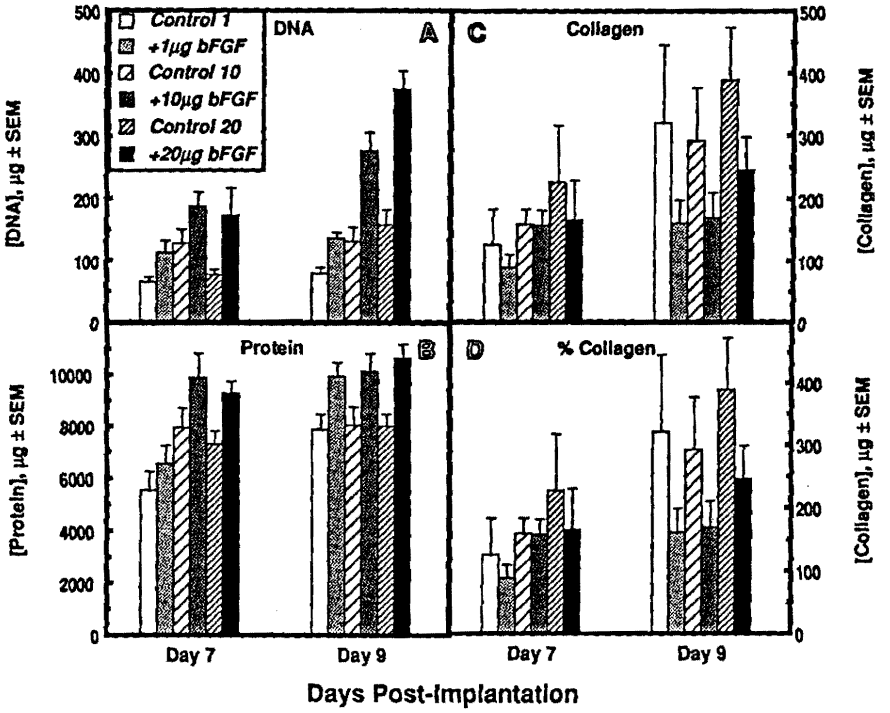


Figure 4

The results of the experiments were encouraging. Figure 4 (28, p. 308) shows the quantitative measurements of wound healing for the sponge implant wound model. Panel A shows the classic mitogenic effect of bFGF, which is its ability to stimulate the replication of DNA. Panel B also shows that bFGF stimulates an increase in protein. Panels C and D show that there was a decrease in the amount of collagen. This is indicative of bFGF's collagenase activity.

Figure 5 (28, p. 310) compares the results of continuous slow release (1.0 micrograms per day) versus acute dosing in the subcutaneous sponge implant model. Although both modes of presentation showed increased granulation and vascularization when compared to the untreated wound, continuous release was shown to produce a much larger angiogenic and mitogenic response than the acute dosage. These findings suggest that the mode of presentation of the growth factor plays a large role in the wound-model healing responses (28, p. 309).

Figure 6 (28, p. 311) illustrates the effects of continuous release of bFGF on the transecting ligament wound model. The treated model showed a marked increase in granulation tissue when compared to the untreated model, but showed no difference in tensile strength. These results are also indicative of bFGF's collagenase activity.

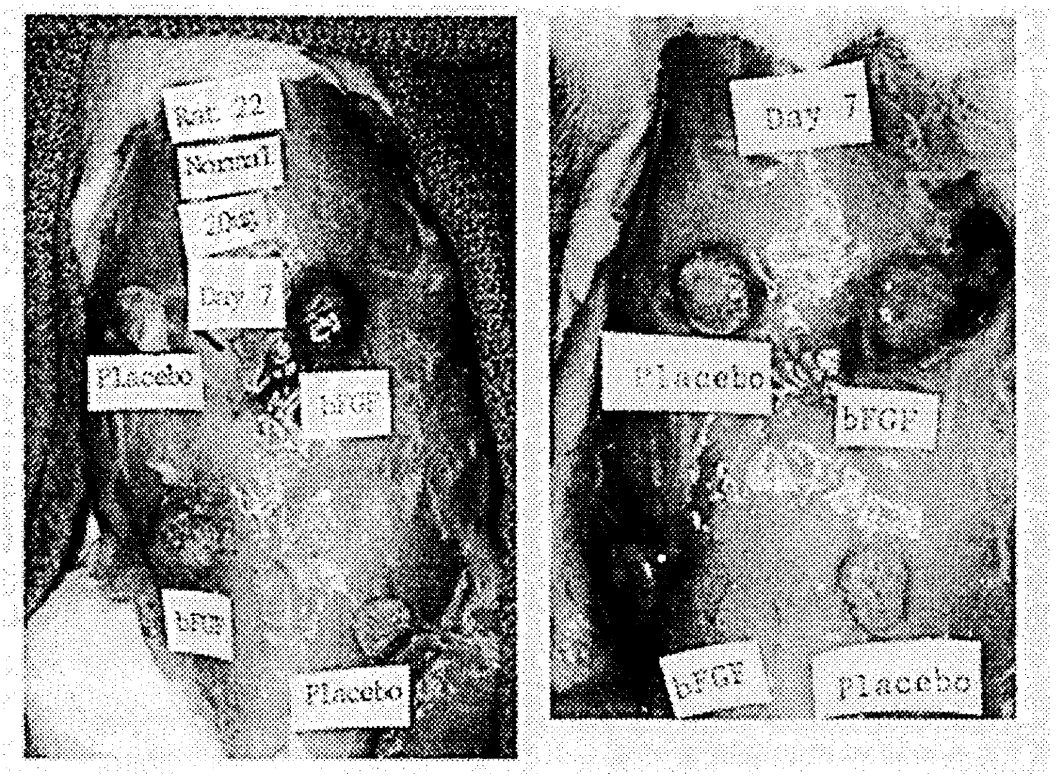


Figure 5

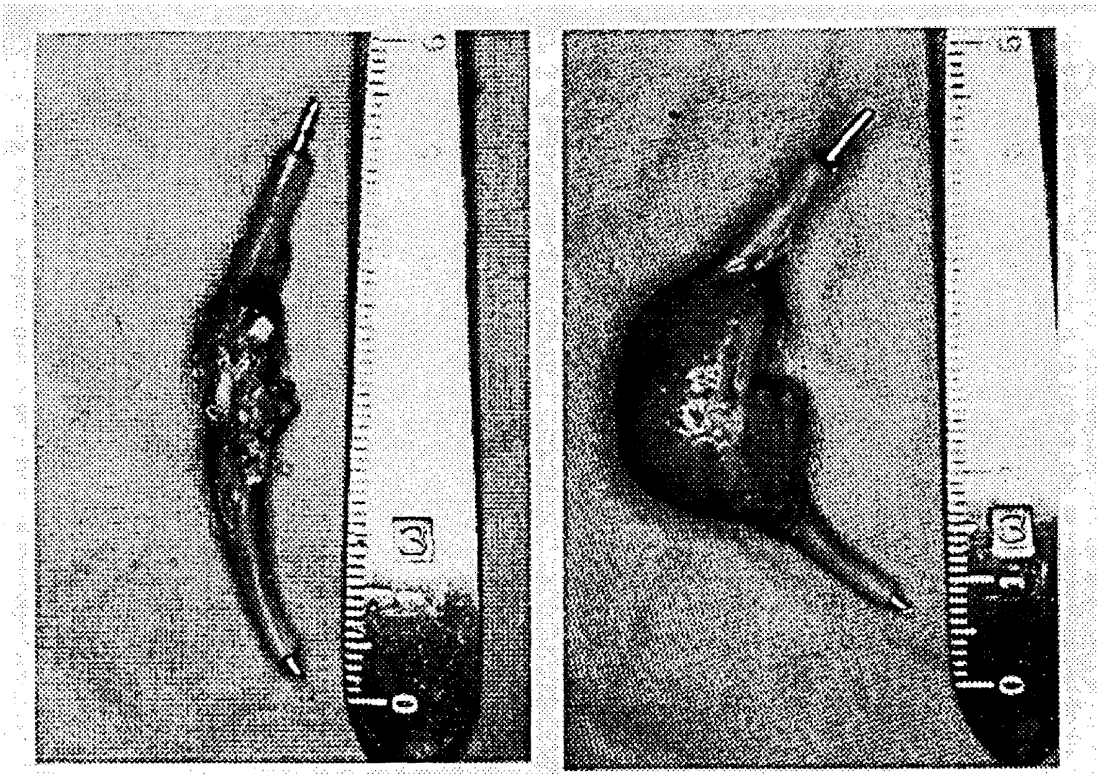

Figure 6

## bFGF and chronic wound healing

The study of bFGF's impact on the repair process in impaired-healing wound models is much more important for a number of reasons. First, normal healing is usually fairly efficient, and not problematic. Growth factors might not accelerate the normal process significantly anyway. Secondly, the most problematic wounds, both clinically and financially, are chronic wounds that are already impaired by things like diabetes, administration of glucocorticoids, exposure to chemotherapeutic substances, or simply old age (28, p. 307). It is the healing of these wounds which would have the most to gain from growth factor research.

In an experiment demonstrated by Fiddes *et al.* (30, pp. 321-328), diabetic mice were used to determine the effects of bFGF on healing-impaired wounds models. Healing rates in diabetic mice are impaired by 35-50% that of a normal mouse (30, p. 321). The mice were either genetically bred to have diabetes or were injected with streptozotocin, which induces diabetes. Diabetic mice were shown to exhibit many of the same symptoms that diabetic humans do, including hyperglycemia, obesity, and hyperinsulemia (30, p. 328).

For the experiment, full-thickness wounds, through the epidermis, dermis and the panniculus carnosus muscle, were made on the flank of each diabetic (db/db) mouse. The bFGF and control solutions were then administered topically to the wounds in 20 microliter acute doses. Healing was measured by contraction, granulization, and epithelialization at the wound site (30, p. 321).

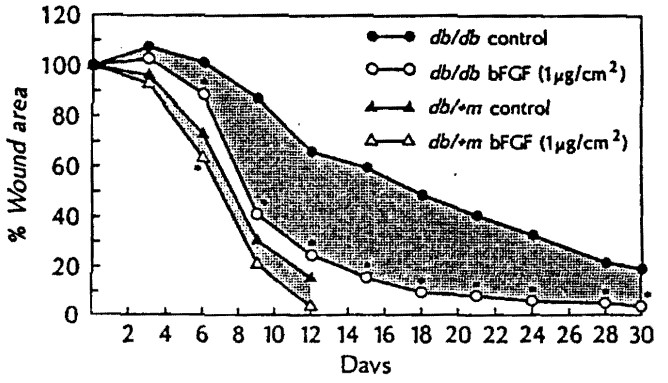


Figure 7

The results, as shown in figure 7, indicate that the time required for a 50% reduction in wound area was improved from 18 days without bFGF to eight days with bFGF (30, p. 323). The graph also shows a much more significant increase in the healing rate of the bFGF-treated diabetic mice than for the bFGF-treated non-diabetic mice. This is evidence that bFGF may be more effective in the clinical setting as a factor to help chronic wound healing, but may not be significantly effective in accelerating the normal wound-healing process. Figure 8 more clearly illustrates that bFGF is effective to help heal diabetic wounds in mice, showing that a diabetic wound treated with bFGF was practically closed by day 12 whereas the untreated wound showed little change (30, p. 324).

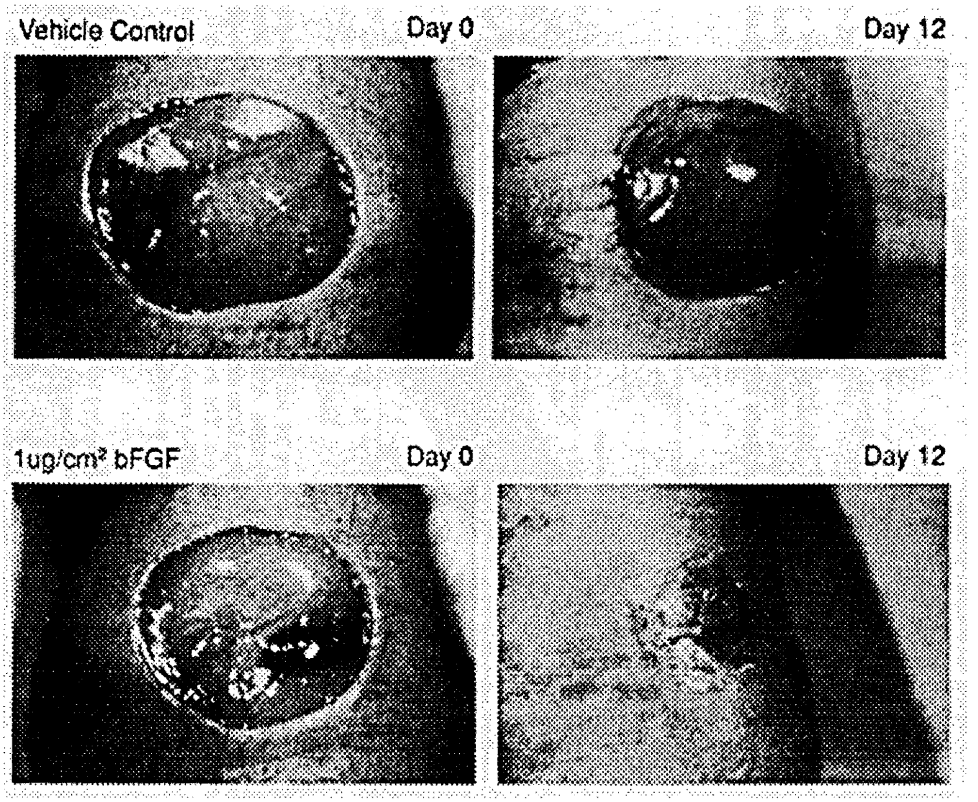


Figure 8

Also studied were the effects of bFGF on bacterial or fungal infection of wounds. This type of study is important because of the recent discovery that topical administration of antimicrobial agents that were previously used to fight infection are now known to be cytotoxic (30, p. 323). Perhaps there is the possibility that bFGF could be used as an alternative therapy. Full-thickness burns were made in the backs of anesthetized rats by placing them into boiling water. The burns were then infected with *E. Coli* bacteria, both acutely and chronically. A dose as small as 1.0 microgram bFGF/cm^2 did improve wound healing in rats that were acutely infected with bacteria. However, chronically infected wounds required a dose of 100 micrograms bFGF/cm^2 to improve healing significantly (30, p. 325). This type of information from various experiments could form the basis of dosage recommendations for the clinical applications of growth factors to different wound types (30, p. 327).

Steroid-impaired models, produced by glucocorticoid injections, showed a 40% decrease in wound healing rate over 12 days. This decrease in the healing rate is due to the inhibition of the inflammatory response. It is important to note that treatment with bFGF was able to restore the normal healing rate (31, p. 452).

**PDGF and chronic wound healing**

PDGF's particular role in the healing process includes the stimulation of compounds like fibronectin, glycosaminoglycan, and collagenase, which are all essential to tissue repair because of their role in the remodeling of the extracellular matrix (27, p. 361). As with bFGF, ex-

periments were done to determine PDGF's effect on the normal healing rate, and similar results were produced. Again, the most important studies were those done on healing-impaired rats.

Of the PDGF isoforms, AA and BB are the best described with respect to their clinical application. Similarities in function, structure, and experimental results may be explained by the fact that both are able to bind to the same receptor type (24, p. 336). In similar experiments with diabetic mice, treatment of wounds with both the AA and BB isoforms of PDGF also resulted in a thickened layer of granulation tissue, capillaries, and fibroblasts (24, p. 334). Treatment of gastrointestinal wounds in mice with PDGF only resulted in an increase in cellularity and migration of macrophages, neutrophils, and fibroblasts at the wound site, but no corresponding increase in wound strength (27, p. 368). These findings are also consistent with PDGF action since it is known to stimulate collagenases.

A study which identifies one of the differences between bFGF and PDGF action involves the treatment of full-thickness wounds with glucocorticoid steroids, which serves to eliminate fibroblasts and macrophages from the wound area. Remember that treatment of this type of wound with bFGF returns the wound's normal healing rate. However, treatment with PDGF shows no effective increase in healing (27, p. 362). Unlike the bFGF mechanism, it is thought that macrophages are essential as a source of PDGF and for the initiation of the signal sequence that touches off a cascade of events culminating in cell proliferation.

### Growth factors and fracture healing

Growth factors have been found to play a key role in the fracture healing process as well. This mechanism, if anything, is more complex than the previously discussed wound repair mechanism in two important ways. First, bone heals by regeneration, not by scarring. Secondly, fracture healing involves the differentiation of osteoblasts and chondrocytes as well as fibroblasts (32, p. 391). bFGF and PDGF are both implicated in the fracture healing process, but are delivered to the site in different ways. PDGF is delivered via platelets in the blood stream, whereas bFGf is delivered through local expression at the fracture site (32, p. 393). Experiments with bilateral fractures of a rat femur provide evidence of growth factor activity. PDGF is shown to appear at the wound site first to stimulate mitogenesis in mesenchymal cells and to regulate the formation of intramembranous bone (32, p. 410). bFGF was shown to be present at the endochondral ossification site because of its known stimulation of collagenase activity, angiogenesis, and mitogenesis of chondrocytes and osteoblasts (32, p. 410). Further studies in the roles of growth factors could lead to new therapies in fracture healing.

## GROWTH FACTOR'S IMPLICATION IN PATHOLOGY

In general, growth factors are clearly beneficial compounds. They are associated with many vital physiological processes including an integral role in wound healing. Ironically, a slight

disruption of the growth factor system may also lead to disaster for an organism. There are several examples where there exists a fine balance between the beneficial adaptation of the growth factor mechanism and the initiation of pathology.

One such example deals with neuronal regeneration in the brain. When bFGF is added to hippocampal cells it is known to increase neuronal survival *in vitro* and *in vivo* (33, p. 221). By using a monoclonal antibody that specifically recognizes bFGF conformation, it was determined that there is a high concentration of bFGF in CA2 neurons in the hippocampus as shown in figure 9 (33, p. 223). This region is also known to be the most resistant to aging and epilepsy indicating that bFGF may contribute to this property (33, p. 222). Alzheimer's Disease

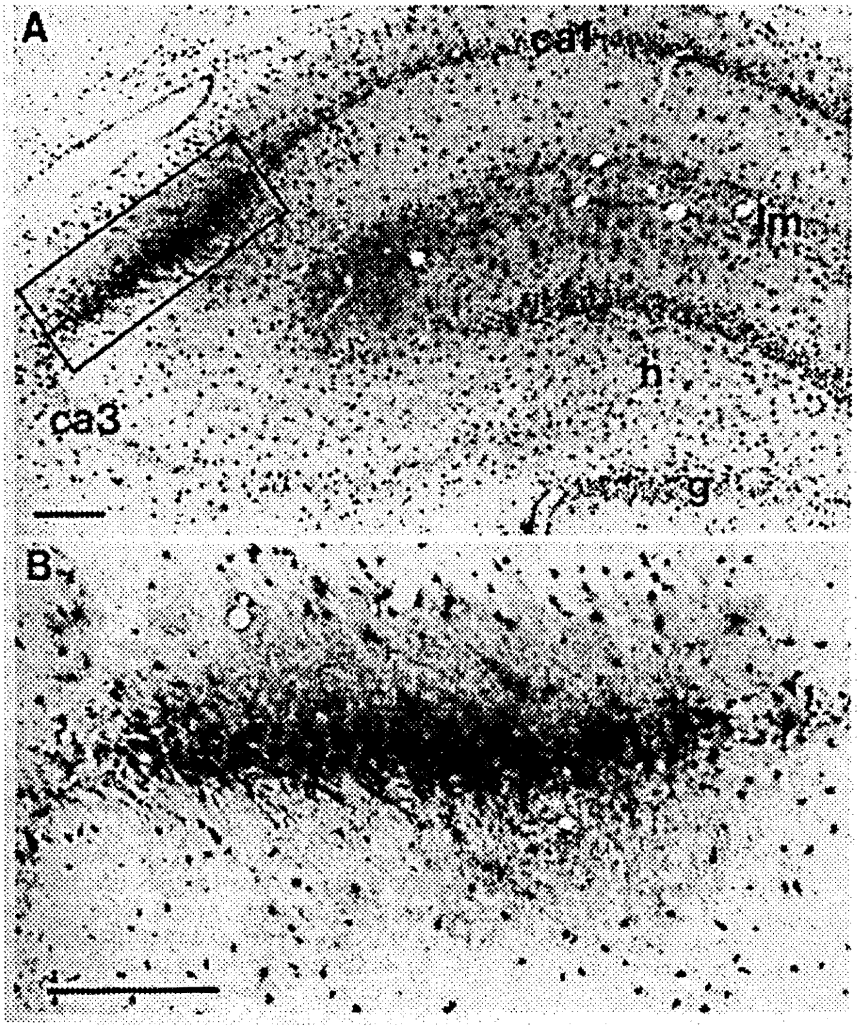


Figure 9

(AD) is a progressive degenerative disease in select brain areas including the entorhinal cortex, which is the main input to the hippocampus and often the first affected. Where neurons first begin to degenerate, bFGF is released in an attempt to sprout new axons to compensate for the loss of connections (33, p. 224). A hallmark of AD is the presence of "senile plaques"

which are deposits of amyloid which appear to form along the border of degenerated areas and neuronal sprouting. The combination of high concentration of bFGF and heparin sulfate may cause the misdirection of sprouting into plaque formation (33, p. 226). Although bFGF initially acts to promote growth and slow degeneration of neurons, it may also lead to the progression of AD if the system is disrupted slightly (33, p. 229).

Huntington's Disease is caused when brain tissue fails to generate enough energy to function. High lactic acid concentrations in the brains of Huntington's patients indicate that their mitochondria are not functioning. (34, p. 292). It was also discovered that there are fewer bFGF receptors in brain cells of Huntington's patients than in normal patients. Since bFGF is known to assist neurons in coping with high energy requirements (34, p. 292), the lack of the normal number of bFGF receptors in conjunction with malfunctioning mitochondria is especially dangerous.

PDGF actions also have a pathogenic side and have been tied to fibrosis and atherosclerosis (22, p. 41). Its activity in the initiation of atherosclerosis is perhaps the most significant example since atherosclerosis may then lead to myocardial and cerebral infarction (35, p. 195). The process is initiated when endothelial cells of the arteries become dysfunctional or injured by things like high cholesterol level, or hypertension (35, p. 195). Injury to the endothelial cells stimulates the attachment of platelets and macrophages and the subsequent secretion of PDGF in an attempt to heal the injury. This causes a cascade of smooth muscle cell proliferation. Eventually the build-up of cells becomes big enough to block the artery and may lead to infarction. Recently, it has been confirmed that PDGF is indeed localized within atherosclerotic lesions (35, p. 196). The same process of injury and subsequent increase in PDGF and macrophages, also occurs within the lungs, leading to pulmonary fibrosis (35, p. 197).

Growth factors also have an undeniable link to cancer. In fact, some oncogenes have been found to have 40-50% homology to bFGF genes (3, p. xi). As mentioned before, some growth factors are only known by their oncogenes. Additionally, more oncogenes are produced as a result of a mutation of the growth factor receptor or compounds associated with growth factor regulation than of the growth factor itself (6). There is evidence that excessive synthesis of, or the production of an altered version of a growth factor or receptor has a transforming effect on recipient cells (4, p. 1159). Cells that are elicited in response to a wound (macrophages, neutrophils, and lymphocytes) might also be attracted to a tumor and may indirectly stimulate angiogenesis of tumor by way of their heparanase activity (18, p. 212). The expression of a normally dormant growth factor gene may also lead to cancer. For example, bFGF is a mitogen for melanocytes, but unlike other cells that react to bFGF, a melanocyte does not produce its own bFGF. The bFGF gene in normal melanocytes is suppressed whereas in malignant melanomas, it is activated (36, p. 233). At the same time, cells that have been damaged by radiation secrete bFGF and are able to repair potentially carcinogenic damage (18, p. 219).

Although growth factor receptors are essential for the transduction of the growth factor signal to the recipient cell, it is also not uncommon for them to be used as portals of entry for

viruses. For instance, the rabies virus is known to enter the cell through acetylcholine receptors (37, p. 1410). There is also strong evidence that the bFGF receptor is used as a portal of entry for the herpes simplex virus type I (HSV I) (37, p. 1410). Inhibitors of bFGF binding and competitive compounds both prevented HSV I uptake. Additionally, some cells lacking the bFGF receptor are known to be resistant to the virus (37, p. 1410).

Although *in vivo* experiments have indicated that growth factors prevent retina degeneration and help heal damaged corneas (3, p. xii), the clinical application of growth factors in eye drops has been problematic because it resulted in the thickening of the cornea (7).

bFGF mRNAs have also been found to be expressed in high levels from cells of Kaposi's sarcoma lesions which are associated with infection by the HIV virus (38, p. 223).

## SOCIETAL ISSUES

### Economic considerations

Growth factors play an important role in the acceleration of the wound healing process, the healing of chronic wounds, and could also be involved in new therapies for incurable pathologies. Thus, the need for the production of synthetic growth factors is evident. Since wounds are as common as the cold and new therapies for existing diseases are always needed, there is also a huge market for synthetic growth factors. A synthetic growth factor, effective in accelerating the chronic wound healing process, would corner much of the wound care market, for which up to four billion dollars is spent annually in the United States alone (1, p. 1065).

The huge market, immense need, and especially the opportunity for financial gain is producing needed cutting-edge research in the areas of growth factor mechanism and synthetic production. Biotechnology companies like Genentech, Amgen, Synergen, Zymogen, and pharmaceutical firms like Merck are all in competition to produce synthetic forms of growth factors (39, p. 141). As of December 1991, however, only Synergen had been granted a patent for recombinant bFGF and is currently seeking market approval from the FDA (39, p. 141). The recombinant human basic FGF (rh-bFGF) is produced by the plasmid pFC81 which carries the bFGF gene into E. Coli for expression (40, p. 330). It is difficult, however, to convince both scientists and FDA officials of a particular growth factor's therapeutic advantage because its effectiveness is not easily quantified. For instance, increasing the oxygen supply, using water-absorbing bandages, or simply cleaning will improve healing of a damaged area (39, p. 141). To prove that the growth factor accelerates the process even further is difficult.

### Ethical issues

Since steroids and growth hormone are also, in a sense, growth factors, one has to ask if either bFGF or PDGF could also be used in potentially dangerous and unethical ways. Could syn-

thetic growth factors get out of hand? Probably not. Since skeletal muscle cells enlarge instead of replicating, and bFGF and PDGF are both mitogens, they could not act to increase skeletal muscle mass. It would also be difficult to increase the entire body size with bFGF and PDGF since their actions are paracrine and autocrine. In order to get a general effect, one would have to apply the growth factors through the blood stream. They would also have to be applied in high doses because the majority would be degraded in the liver. High doses could have potentially dangerous side effects such as unregulated cell growth. However, the growth would probably be localized and non-malignant (6).

The ethical considerations of patenting should also be considered. Should we be allowed to patent synthetic growth factors? After all, growth factors are a natural part of the human body. Is it ethical to patent parts of ourselves? If so, where does it stop? For instance, who is going to own the patent for the life process itself? There have already been reports of obvious misuse of the patent system. In particular, one scientist requested patents on peptide sequences discovered in the brain without even knowing their function! James Watson, of Watson and Crick fame, was so opposed to the random patenting of sequences, that he lost his job with the human genome project (6). After all, once a patent for a particular growth factor has been received, rival labs could request a patent on a new growth factor with a similar function simply by altering a single amino acid. Conversely, if patents on growth factors were not permitted, perhaps it would decrease the initiative to produce these potentially beneficial products

## FUTURE OUTLOOK

One would think that the combination of "cutting-edge research, a crying medical need, and a huge market" would be a "surefire prescription for success (1, p. 1065)." Results, however, have been disappointing mainly because researchers are trying to find a single "cure-all" growth factor (1, p. 1065). Since there are an enormous number of known growth factors in tissues, it has been extremely difficult to determine which ones are most crucial to wound repair and which ones are essential to the developmental process. There are a number of areas to which growth factor research might be directed in the future.

The use of growth factor synergism, or the interaction of two or more growth factors, has shown promise and is definitely more representative of what happens *in vivo* (28, p. 313). A veritable cocktail of growth factors which act in different ways might produce a more desirable clinical effect. For instance, the experiments discussed showed that bFGF and PDGF both increased granulation tissue but because of their collagenase activities did not increase collagen content or tensile strength of the healed wound. The activation of transforming growth factor (TGF) is necessary to induce collagen type I synthesis. Therefore, a suggestion might be to give PDGF to recruit infection-fighting cells, bFGF to promote vascularization, and TGF to help deposit and strengthen the matrix.

Also, the type of growth factor that is used in clinical situations should depend upon the type of wound (39, p. 141). For instance, wounds that have circulation disorders like diabetic or venous ulcers and pressure sores might be most efficiently treated with bFGF because of its known promotion of angiogenesis.

Improving the quality and not just the speed of the wound healing process is another interesting area of research for the future. This might involve the study of wound healing in fetuses. Children that had been operated on as 18 to 28 week old fetuses have shown no trace of a scar upon birth (1, p. 1066). Histologically, wounds that are repaired fetally and normal skin are practically indistinguishable (1, p. 1066). Perhaps the study of the factors associated with fetal wound healing could lead to the ability to heal wounds without a scar. This would have a major aesthetic impact as well as alleviating many of the problems associated with scar tissue.

Moreover, future research might be directed towards the production of growth factor "antagonists" which could be beneficial where the stimulation of growth factors was uncontrolled (1, p. 1065).

## SUMMARY

Perhaps one of the most important aspect of growth factors is their role in the wound healing process. Experiments have shown that synthetic growth factors play an essential role in the healing of chronic wounds and the acceleration of the normal healing process. Although growth factors are known to be associated with vital physiological processes like mitogenesis, embryonic development, angiogenesis, and neuron regeneration, they are also implicated in potentially lethal pathologies like Alzheimer's Disease, melanomas, and atherosclerosis. The potential for clinical applications of growth factors and the huge market for such a product has produced intense research and competition among biotechnology companies to corner the market on synthetic growth factors. In conclusion, it is important to realize that a major part of the growth factor mechanism and methods of interaction with both the producing and recipient cells is still unknown. A vast amount of research still remains to be done in order to clarify the growth factor's possible impact on clinical application. An appropriate statement is that, "If you aren't confused, you simply don't understand what's going on here (8, p. 228)."

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# ADDENDUM

## Introduction

The FGF family consists of seven related polypeptides of which bFGF is one of the best characterized. Some of bFGF's *in vivo* functions include mitogenesis of a wide variety of cell types, chemotaxis, cell differentiation, and angiogenesis. These properties have made bFGF valuable as a synthetic product for clinical applications in chronic wound healing, accelerating the normal healing process, and perhaps even as a new therapy for diseases like Alzheimer's disease or atherosclerosis. Although bFGF is primarily a mitogen, it is also functions to increase the life span of cultured cells, and to prevent the death of cells that are exposed to a serum-free medium. Through experiments with cultured, non-transformed 3T3 mouse cells, I hope to show how long it takes bFGF to retrieve cells from death, and to suggest a possible mechanism for such a retrieval.

## Materials and Methods

Non-transformed mouse 3T3 fibroblast cells were cultured in a medium with 10% calf-serum and exposed to radioactive Chromium 51 (Cr 51). After six hours, the cells were rinsed with serum-free medium. Half of the cultures were then exposed to a serum-free medium with bFGF while the control cultures were exposed only to serum-free medium. At 5, 10, and 15 hours, all cultures were assayed with a scintillation counter for the percent chromium released.

## Results and Discussion

Although Cr 51 may also slowly leak out of the cells in both the control and bFGF-treated cultures, the percent Cr 51 released is generally a measure of cell death. The experiment was reproduced nine times and the average percent releases, as shown in the table and graph, indicate that a significant difference between the survival of the control and the bFGF-treated cultures occurs between 5 and 10 hours. Since bFGF is known to produce cell proliferation only after a 10-12 hour lag period, the signal transduction for the retrieval of cells from death must be different from that of mitogenesis. Although the two functions of bFGF clearly differ in their signal transduction, perhaps they are similar. For instance, since bFGF signal transduction is known to involve the phosphatidylinositol pathway, activation of different parts of the pathway may result in the production of multiple signals with variable functions. Some early response genes, which are activated 5 hours after exposure to bFGF, may be essential in initiating the mechanism for cell-death retrieval, whereas the delayed response genes may be critical for the cell proliferation mechanism. It is known that 3T3 cells release certain proteins within 8 hours of exposure to bFGF. Perhaps these proteins serve as a self-defense mechanism and protect against cell death.

**Results of Cr51 Release Assay**

Average Percent Cr51 Retained

| Time | Plus FGF | Minus FGF |
|---|---|---|
| T0 | 100 | 100 |
| T5 | 89.53 | 90.15 |
| T10 | 86.2 | 80.5 |
| T15 | 81.5 | 69.6 |